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Growth temperature influences postharvest glucosinolate concentrations and hydrolysis product formation in first and second cuts of rocket salad

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ABSTRACT

Rocket salad species (*Diplotaxis tenuifolia* and *Eruca sativa*; also known as *E. vesicaria*) are known for their high concentrations of health-related isothiocyanates, which are derived from secondary metabolites called glucosinolates. Increases in temperature due to climate change and extreme weather event frequencies over the coming decades are likely to influence not only the growth of leafy vegetables, but also their nutritional density. It is therefore essential to determine the impacts of these in order to mitigate crop losses and nutritional decline in future. Our data show there is a strong influence of pre-harvest growth temperatures on glucosinolate biosynthesis and formation of glucosinolate hydrolysis products postharvest, and that this is genotype dependent. High growth temperature (40 °C) severely retarded germination, growth, regrowth, and survival of rocket plants. Highest glucosinolate concentrations were observed in first and second cuts at 40 °C, but did not correspond to highest isothiocyanate concentrations (observed at 30 °C, second cut). Hydrolysis product formation is proportionately not as great as glucosinolate increases at 40 °C, possibly due to inhibition of enzyme function(s) at higher temperatures. These data indicate that high growth temperatures increase glucosinolate accumulation, but growth and productivity is significantly reduced. Much greater emphasis is needed for breeding cultivars tolerant to high growth temperatures in order to maximise nutritional benefits imparted by temperature stress.

1. Introduction

Rocket salads are a popular group of leafy vegetables belonging to the Brassicaceae family. *Diplotaxis tenuifolia* and *Eruca sativa* comprise the majority of global rocket production, and are well known for pungent aromas and flavours. Each species has distinct morphological characteristics, though *E. sativa* is much more varied in this regard. Over the last 20 years there has been a surge in interest in the crops for their phytochemical content, particularly for glucosinolates (GSLs) and their hydrolysis products (GHPs; Bell and Wagstaff, 2019). Foremost of these are isothiocyanates (ITCs), and specifically sulforaphane (SF); consumption of which has been linked with a reduced risk of developing some cancers (Fimognari and Hrelia, 2007).

GSL profiles are notoriously variable across growth environments in many Brassicaceae species, and the formation of GHPs even more so (Bell and Wagstaff, 2019). GSL biosynthesis is inherently tied to the stress responses of all Brassicales plants (Mostafa et al., 2016), and as such, concentrations within tissues can vary markedly according to growth temperature (Kask et al., 2016), light quality (Schreiner et al., 2009), and salinity (Cocetta et al., 2018); as well as biotic factors from pests and disease (Schlaeppi et al., 2010). Climate change is leading to more extreme temperatures in places used to cultivate horticultural crops, and consumer demand is leading to the adoption of more land and more protected cultivation practices to meet the yield and quality expectations.

A large amount of work has been done to determine the glucosinolate (GSL) profiles of rocket within first harvest (or cut) leaves (Cataldi et al., 2007; Chun et al., 2015; Force et al., 2007; Toledo-Martín et al., 2017), and only one obscure study has assessed second cut composition (Nitz and Schnitzler, 2002), but only looked at three compounds. Second cuts are primarily favoured by growers and processors for their perceived increased pungency and overall quality (Bell and Wagstaff, 2019), yet the scientific literature has thus far failed to consider this common horticultural practice in experimental designs. It is speculated that multiple cuts increase the abundance of glucosinolates and isothiocyanates in rocket species. The increase of secondary metabolites in response to mechanical wounding is well known in other horticultural species (Jahangir et al., 2009). This has clear implications for taste, flavour, and health-related properties of leaves.

The impact of different growing environments, such as temperature

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variation, on crop nutritional composition is also poorly studied in rocket; adding an additional influencing factor on these traits. It is therefore possible that variable growing temperatures will also affect (positively or negatively) the consumer eating experience. The exact genetic mechanisms that regulate GSL biosynthesis under high or low temperatures are unknown, partly due to interacting and co-occurring stresses, such as drought. It is likely however that the imposition of high/low temperature stress promotes activity of transcription factors such as *MYC2* and *MYB28*, which promote GSL biosynthesis (Gigolashvili et al., 2009).

Temperature effects upon GSL synthesis and GHP formation are poorly understood in rocket species, and have important implications for the synthesis of specific health-associated compounds. Rocket crops are grown on every inhabited continent, and are exposed to a huge range of cultivation temperatures. They can be grown under mild, temperate conditions, such as in southern England, (summer maximum temperatures averaging 20.4 °C; Met Office 1981–2010 data), to hot Mediterranean temperatures (such as the Bay of Naples, Italy, summer maximum temperatures averaging 29.5 °C; World Meteorological Organization). In addition, crops are commonly cultivated under glass or polytunnel in summer months, where internal daytime temperatures can rise to over 35 °C (Di Gioia et al., 2018). In growing regions such as Lazio (Italy) and New South Wales (Australia), outdoor summer daytime temperatures can regularly exceed 40 °C, and therefore have significant impacts on the growth of leafy vegetables. Crops under protection are therefore doubly affected, as internal temperatures may exceed 50 °C without adequate ventilation. By the end of the 21st century, atmospheric CO_2 concentrations are projected to rise to between 730 and 1000 ppm. This will lead to average global temperature increases of between 1–3.7 °C (Gray and Brady, 2016). Combined with an increased likelihood of extreme weather events (such as heat waves and drought), protected leafy crops such as rocket are especially vulnerable to losses and or changes in growth rate.

The effects of such growing extremes are presently unknown, and it is likewise unstudied how growth temperature affects regrowth, phytochemical content, or shelf life retention of health-associated compounds. Postharvest work has already demonstrated that these compounds are subject to fluctuation (Bell et al., 2017c; Yahya et al., 2019), but it is unknown to what degree growth temperature and cut influence this process. In light of climate change and global warming effects in future, it is also likely that extremes in temperature will become more common, and therefore it is important to understand how crop growth and quality may be affected.

This study presents phytochemical data relating to the growth of two *D. tenuifolia* and two *E. sativa* rocket cultivars under different growth temperatures. This study is the first to examine the impact of cultivation temperatures on secondary metabolite formation that has consequences for nutrition and flavour qualities of rocket crops. We hypothesised that each species would see increases in GSL concentrations at the higher cultivation temperatures, but that each cultivar would produce differing relative concentrations according to genotype, as has been highlighted in a previous study (Bell et al., 2015). We speculated that the initial concentrations of GSLs postharvest would influence the degree of biosynthesis and retention during the cold storage period, and in turn impact the abundance of GHPs formed.

2. Materials and methods

2.1. Plant material

Two *D. tenuifolia* and two *E. sativa* pre-commercial cultivars were supplied by Elsoms Seeds Ltd. (Spalding, UK). For reasons of commercial sensitivity specific details regarding the genetic origin of these will not be given. *E. sativa* cultivars were designated RS4 and RS8, and *D. tenuifolia* cultivars RW2 and RW3.

Table 1
Numbers of biological replicates under each temperature condition and cut per temperature treatment.

Cultivar	Temperature condition & cut					
	20 °C		30 °C		40 °C	
	1st	2nd	1st	2nd	1st	2nd
RS4	12	12	12	7	16	dns
RS8	11	11	15	8	13	6
RW2	5	3	9	3	dns	dns
RW3	10	7	10	7	2	dns

Abbreviations: RS = salad rocket; RW = wild rocket; dns = did not survive.

2.2. Growing conditions, simulated processing, and shelf life storage sampling

Forty seeds of each cultivar were sown into module trays containing peat-based seedling compost, and germinated under three temperature conditions in Saxcil growth cabinets. The three temperature conditions were as follows: 20 °C (daytime; 15 °C night), 30 °C (daytime; 25 °C night), and 40 °C (daytime; 30 °C night). Lighting conditions were consistent between each chamber and set to a long-day cycle (16 h light, 8 h dark). Light intensity was set to 380 μmol m^{-2} s^{-1}. Humidity was ambient. Healthy seedlings were transplanted into 1 l pots (containing peat-based compost) on an individual basis, upon the development and expansion of two true leaves. Pots were watered daily, as required, to capacity.

Plants were harvested on an individual basis, and were considered of commercial maturity once 10–15 leaves were developed. See Table 1 for numbers of biological replicates harvested for each cultivar under each respective condition. Upon reaching this point, plants were harvested by hand using sterile scissors and left to regrow. It should be noted that not all plants survived the first cut, and that the 40 °C treatment severely impaired growth and survival.

All plants were harvested between 10 a.m. and 12 p.m to minimise the effects of diurnal fluctuations in secondary metabolites (Huseby et al., 2013). The harvested leaves were initially placed in Ziploc bags and then transferred to the laboratory. Upon arrival, plants were hand processed individually by turbulent washing in mildly chlorinated water (30 ppm, sodium hypochlorite; Suslow, 2000) for one minute, followed by gentle rinsing in non-chlorinated water for one minute. Finally leaves were placed in a hand operated salad spinner and dried for another minute. Leaves were divided into equal amounts and designated D0, D4, and D7 according to the beginning of shelf life storage. D0 samples were placed immediately into a -80 °C freezer. D4 and D7 samples were placed in laser perforated bags and closed with an electric heat-sealer, then stored in the dark at 4 °C (Bell et al., 2016). On respective sampling days, each of the bagged leaves were frozen at -80 °C. All sampling took place between 10 a.m. and 12 p.m, as per the initial time of harvest. This entire process was repeated for the second cut of regrown leaves until all plants were either harvested or had died.

2.3. Leaf material preparation, and extraction

Frozen leaf material was lyophilized in batches for three days. Leaves were ground into a fine powder using a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ, USA) and stored in tubes until extraction and analysis.

GSL extraction was performed as per the protocol presented by (Bell et al., 2015) with modifications. Briefly, 40 mg of dried leaf powder was placed into Eppendorf tubes and put into a heat block (80 °C for ten minutes). Afterwards, 1 ml of preheated methanol water (70 % v/v) was added to dried powder, vortexed vigorously, and placed in a water bath

(75 °C) for 20 min. Samples were cooled to halt the extraction and then centrifuged at full speed for five minutes at room temperature (~22 °C), the supernatant collected, and filtered (0.22 µm PVDF Acrodisc syringe filters; VWR, Lutterworth, UK). Crude extracts were stored at -80 °C before dilution (5x) and analysis conducted by LC–MS.

GHPs were extracted according to the protocol published by Ku et al. (2016) with modifications. The extraction duration was optimized for maximum yields of GHPs by comparison of extractions for three hours incubation at 30 °C with immediate dichloromethane (DCM) extraction, and three, nine, and 21 h post incubation with DCM.

50 mg of sample was hydrolysed in 1 ml of d.H_2O for three hours at 30 °C, before subsequent extraction in dichloromethane (DCM) overnight (21 h). The DCM layer was then collected and transferred to glass vials and stored at -80 °C until analysis by GC–MS.

2.4. LC–MS and GC–MS analyses

For LC–MS, samples were analyzed in a random sequence with standards and QC samples. External standards of progoitrin (PRO; 99.07 %, HPLC), glucoraphanin (GRA; 99.86 %, HPLC), glucoerucin (GER; 99.68 %, HPLC), glucobrassicin (GBR; 99.38 %, HPLC), and gluconasturtiin (GNAS; 98.38 %, HPLC) were prepared for quantification of GSL compounds according to the method presented by Jin et al. (2009). GER was used to quantify glucorucolamine (GRM), diglucothiobeinin (DGTB), glucosativin (GSV), and DMB, as no standards are presently available for these compounds. GBR was used to quantify the indole GSLs 4-methoxyglucobrassicin (4MOB) and neoglucobrassicin (NGB; Table 2). All standards were purchased from PhytoPlan (Heidelberg, Germany). Recovery of extracted GSLs was calculated by spiking six random samples with sinigrin upon the addition of pre-heated methanol (Merck, Gillingham, UK). The average recovery of sinigrin was 104.8 %. Limits of detection (LOD) and quantification (LOQ) were established for the method by running serial dilutions of sinigrin (LOD = 5.38 µmol L^{-1}; LOQ = 16.3 µmol L^{-1}).

LC–MS analysis was performed in the negative ion mode on an Agilent 1260 Infinity Series LC system (Agilent, Stockport, UK) equipped with a binary pump, degasser, auto-sampler, column heater and diode array detector; coupled to an Agilent 6120 Series single quadrupole mass spectrometer. Separation of samples was achieved on a Gemini 3 µm C18 110 Å (150 × 4.6 mm) column (with Security Guard column, C18; 4 mm x 3 mm; Phenomenex, Macclesfield, UK). GSLs were separated during a 40 min chromatographic run, with a 5 min post-run sequence. Mobile phases consisted of ammonium formate (0.1 %; A) and acetonitrile (B) with the following gradient timetable: (i) 0 min (A–B, 95:5, v/v); (ii) 0–13 min s (A–B, 95:5, v/v); (iii) 13–22 min s (A–B, 40:60, v/v); (iv) 22 − 30 mins (A–B, 40:60, v/v); 30 − 35 mins (A–B, 95:5, v/v); (v) 35 − 40 mins (A–B, 95:5, v/v). The flow rate was optimized for the system at 0.4 ml min^{-1}, with a column temperature of 30 °C; 20 µl of sample was injected into the system. Quantification was conducted at a wavelength of 229 nm.

MS analysis settings were as follows: Atmospheric pressure electrospray ionization was carried out in negative ion mode (scan range *m/z* 100–1000 Da. Nebulizer pressure was set at 50 psi, gas-drying temperature at 350 °C, and capillary voltage at 2000 V. Compounds were identified using their primary ion mass [M-H]- (Cataldi et al., 2007) and by comparing relative retention times with those of Lelario et a. (2012; Table 2). Data were analyzed using Agilent OpenLAB CDS ChemStation Edition for LC–MS (vA.02.10). GSL concentrations from each time point were averaged; see Table 1 for all *n* per treatment. This approach was also conducted for GHP analysis.

GHPs were identified and analysed according to the method presented by Bell et al. (2017) with the following modification. Extracts were separated on a Zebron ZB-AAA (10 m, 0.25 mm i.d.; Phenomenex) capillary during a seven minute run. GC conditions were as follows: split 1:20 at 250 °C, with a 2.5 µl injection. Helium was used as the carrier gas at a flow rate of 1.5 mL min. The oven program was: 30 °C min from 110 °C to 320 °C, with a one minute hold at 320 °C. Concentrations of all GHPs were calculated as equivalents of SF standard (Sigma).

All concentrations quoted within the text are on a dry weight basis for both GSLs and GHPs.

Table 2
Glucosinolates and glucosinolate hydrolysis products identified in *Eruca sativa* and *Diplotaxis tenuifolia* cultivars.

Glucosinolates					
	Trivial name	Abbreviation	R-group name	Retention time	Identifying *m/z* $[M-H]^-$
1	Glucorucolamine [b]	GRM	4-(cysteine-*S*-yl)butyl	4.7	493
2	Progoitrin [a]	PRO	(2*R*)-2-hydroxybut-3-enyl	5.9	388
3	Glucoraphanin [a]	GRA	4-methylsulfinylbutyl	6.0	436
4	Diglucothiobeinin [b]	DGTB	4-(β-D-glucopyranosyldisulfanyl)	12.7	600
5	Glucosativin [b]	GSV	4-mercaptobutyl	16.4	406
6	Glucoerucin [a]	GER	4-methylthiobutyl	22.7	420
7	–	DMB	Dimeric 4-mercaptobutyl	23.0	405 (811, 731)
8	Glucobrassicin [a]	GBR	Indol-3-ylmethyl	23.5	447
9	Gluconasturtiin [a]	GNAS	2-phenethyl	24.0	422
10	4-methoxyglucobrassicin [c]	4MOB	4-methoxy-3-indolylmethyl	24.2	477
11	Neoglucobrassicin [c]	NGB	1-methoxy-3-indolylmethyl	25.6	477

Glucosinolate hydrolysis products			
	Trivial name	Hydrolysis product of	MS spectra *m/z* (base ion in bold)
1	Sativin [d]	Glucosativin	147, **114**, 87, 72, 60
2	Erucin [d]	Glucoerucin	161, **115**, 72, 61
3	Sulforaphane [a]	Glucoraphanin	177, **160**, 114, 72, 55
4	Bis-(4-isothiocyanatobutyl)-disulfide [d]	DMB	292, 146, **114**, 87, 72, 55

[a] = Quantified using authentic standards.
[b] = Quantified using glucoerucin standard.
[c] = Quantified using glucobrassicin standard.
[d] = Quantified using sulforaphane standard.

2.5. Statistical analysis

ANOVA analyses of all data were performed using XL Stat (Addinsoft, Paris, France). Each respective analysis was conducted with a protected post hoc Tukey's Honest Significance Difference (HSD) test ($P < 0.05$). The Type III Sums of Squares significance values (at 5 %, 1 %, and 0.1 % thresholds) and summary pairwise comparisons tables for GSL and GHP data are available in Supplementary Data File S1.

Principal Component Analysis (PCA) was performed using XL Stat with Pearson correlation analysis (*n*-1), Varimax rotation, and the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy. The KMO value for the analysis was 0.71, indicating a satisfactory level of sampling. The analysis produced four informative Principal Components (PCs) with Eigenvalues > 1.0. The cumulative explained variability within these components totalled 78.1 %. After Varimax rotation, PCs 1, 2, and 8 produced the highest degree of explanatory spatial separation.

3. Results and discussion

3.1. The effects of growth temperature on germination and time to first and second cuts

3.1.1. Germination

Germination time was shortest under the 30 °C condition, and *E. sativa* cultivars showed a clear trend for earlier establishment than *D. tenuifolia* (Fig. 1a). RW2 and RW3 had slow germination and growth at both 20 °C and 40 °C, with RW2 not germinating at all under the latter condition. As rocket species have a Mediterranean origin, it is not entirely surprising that germination is optimal at 30 °C, however our data do suggest that *E. sativa* is better adapted to temperature extremes. This could be of particular relevance to growers cultivating rocket under glass or polytunnel where temperatures may regularly exceed 35 °C in the summer. Conversely, growers in cool or temperate regions, cultivating rocket in open field, may find *E. sativa* quicker to establish.

3.1.2. Time to first cut

Harvest age of rocket is highly variable between growth environments (Hall et al., 2015), and as such we selected a physiological growth phase as a benchmark for harvest between each temperature condition (development of 10–15 leaves). At 40 °C all plant growth was severely retarded (Fig. 1b), and no plants were ready for first harvest before 40 days. This suggests that adverse high temperature conditions may have a large impact on the productivity of rocket crops, regardless of species.

Exposure to temperatures > 37 °C for prolonged periods of time can be lethal in many plants without acclimatisation. This is due to the inactivation or denaturation of proteins (Schöffl and Panikulangara, 2018). Without adequate time to adjust to heat shock (such as through expression of heat shock proteins) growth is slowed or even halted; ultimately leading to plant death. It is therefore remarkable that the rocket plants (particularly RS8) tested in this experiment were able to tolerate these conditions.

3.1.3. Time to second cut

The differences between temperature conditions for the second harvest were less pronounced (Fig. 1c). At 40 °C however there was no regrowth for RS4, RW2, or RW3. Plants of these cultivars typically senesced a few days after first cut and died. As mentioned previously, given that the 40 °C temperature was extreme (Schöffl and Panikulangara, 2018), *E. sativa* in particular displayed a high tolerance.

Such conditions are not unheard of in protected environments, and may become more common under protected conditions in future due to global warming. It is interesting therefore that RS8 showed little adverse effects to the extreme temperature condition, and regrew within approximately 15 days. While rocket is not bred for temperature tolerance at this time, it does suggest that if climatic conditions become more challenging in future there are cultivars capable of withstanding such high temperature extremes. As will be discussed in subsequent sections, this may also have important implications for biosynthesis of health-related compounds in rocket.

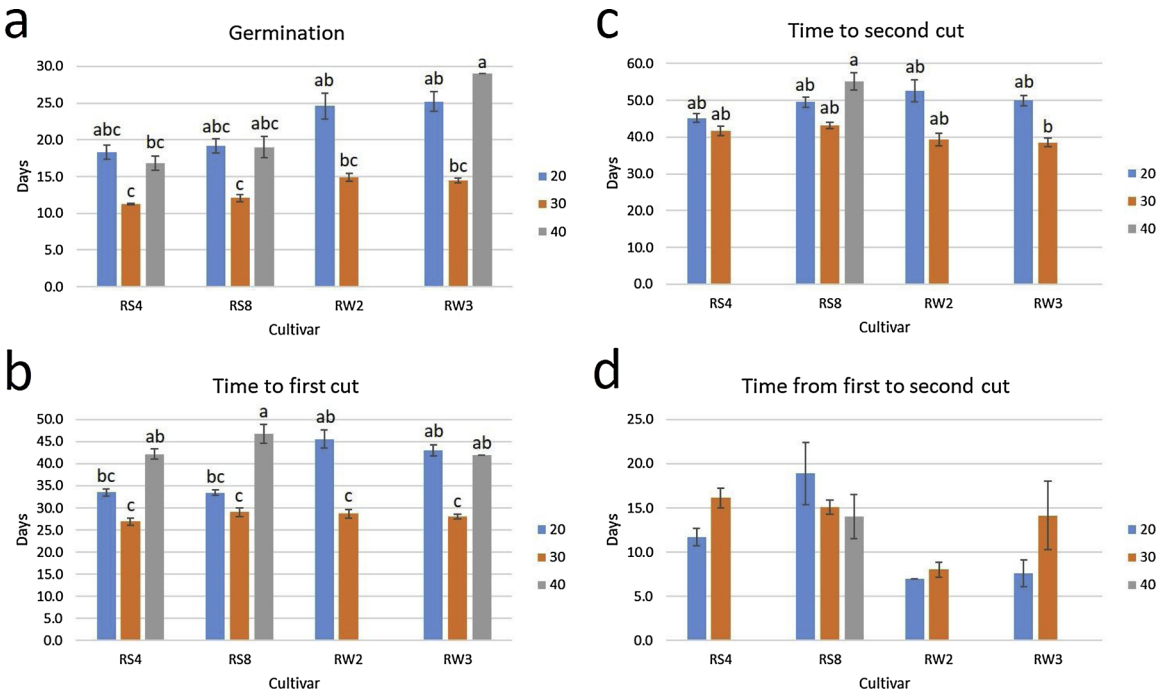

Fig. 1. The number of days taken for *Diplotaxis tenuifolia* (RW) and *Eruca sativa* (RS) cultivars to germinate (a), reach first cut maturity (10 – 15 true leaves; b), reach second cut maturity (10 – 15 regrowth leaves; c), and from first to second cut (d). Error bars represent standard error of the mean. See Table 1 for the numbers of replicates for each cultivar and treatment.

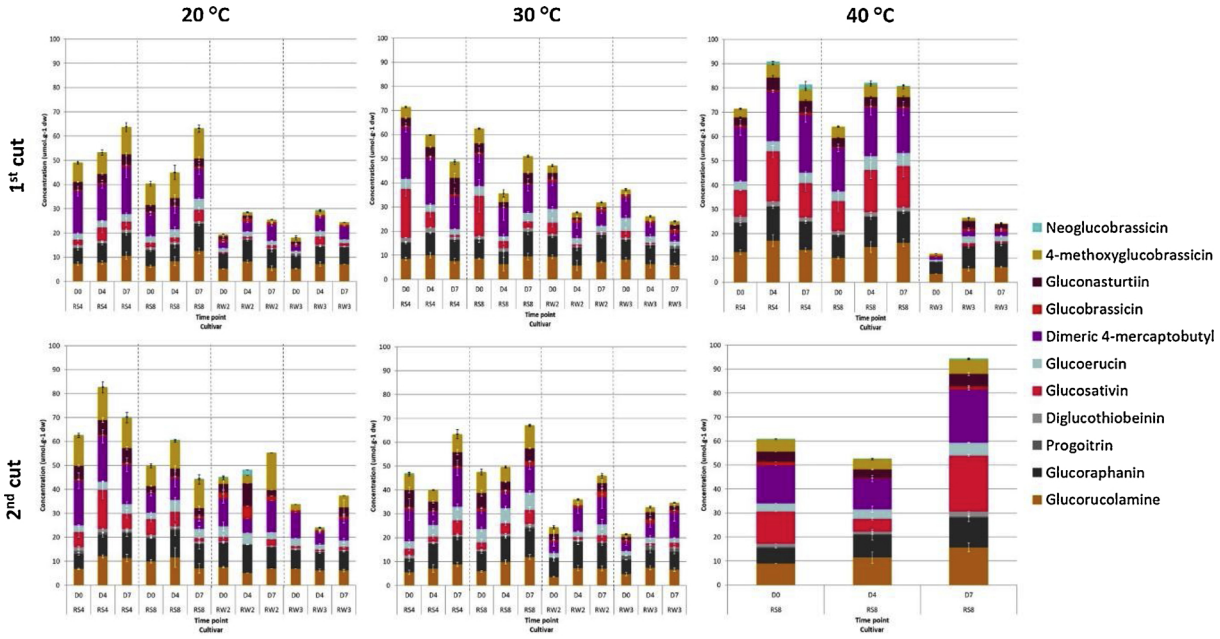

Fig. 2. Glucosinolate concentrations in first and second cut *Diplotaxis tenuifolia* (RW) and *Eruca sativa* (RS) grown at four different temperatures. See inset for glucosinolate colour coding. Abbreviations: D0, start of shelf life; D4, fourth day of shelf life; D7, seventh day and end of commercial shelf life. Error bars represent standard error of the mean for each respective compound. For detailed statistical and Tukey's HSD pairwise comparisons, see Supplementary Data File S1. See Table S1 for the numbers of replicates for each cultivar and treatment. Note that sample RW3 at 40 °C consists of < 3 biological replicates due to plant death.

3.1.4. Time from first to second cut

Regrowth of RW2 was extremely fast, taking only seven days to regrow > 10 leaves after the first cut at 30 °C (Fig. 1d). These data suggest that *D. tenuifolia* second cuts may be much more productive than *E. sativa* under 20–30 °C conditions. While initial establishment and growth rate is slow in the first cut, this is offset by a much quicker subsequent regrowth rate. Leaves were however much smaller than at first cut, and would likely be much lower yielding (even at high planting densities) than *E. sativa* second cuts.

3.2. The effects of growth temperature on glucosinolate concentrations and hydrolysis product formation during shelf life storage

3.2.1. First cut at 20 °C

There was a clear trend in first cut *E. sativa* cultivars for higher GSL accumulation compared with *D. tenuifolia* (Fig. 2). At D0, ANOVA pairwise comparisons of these samples were non-significant between cultivars, with the exception of 4MOB ($P < 0.0001$; Table 3 & Supplementary Data File S1). RS4 and RS8 both showed a clear trend for increased GSL concentrations over the seven day shelf life period, peaking at the final time point (D7). This is in agreement with observations made by Bell et al. (2017) in a field grown UK *E. sativa* crop. RW2 and RW3 by comparison peaked on D4, but contained almost half the concentrations of RS4 and RS8. These trends were only followed by RS8 and RW2 for hydrolysis product formation, and concentrations were generally low across all time points (Fig. 3). This has implications for consumers, and suggests that health-related benefits are cultivar and time-dependent postharvest. The variability of isothiocyanates and other GHPs in rocket postharvest is documented (Bell and Wagstaff, 2019). The timing of consumption is therefore a critical consideration for determining the efficacy of rocket cultivars against disease and chronic illness, and should be included as a more prominent factor for consideration in clinical investigations.

3.2.2. Second cut at 20 °C

In the second cut at 20 °C the trend between species was reversed: RS4 and RS8 peaked on D4, and RW2 and RW3 peaked on D7 for GSLs (Fig. 2). Again, indolic GSL concentrations were significantly different at D0. RW2 contained significantly higher abundance of GBR than RS4 and RS8 ($P = 0.003$), and conversely, RS4 had higher amounts of 4MOB compared with each of the *D. tenuifolia* cultivars ($12.7 \pm 0.9\,\mu mol\ g^{-1}$; $P < 0.0001$). These differences were repeated in D4 samples (GBR and 4MOB, $P < 0.0001$, Table 3 & Supplementary Data File S1) and suggest a distinct difference in indolic GSL metabolism between the two species; something that has not been previously observed. Indolics are linked with chemopreventative properties, such as promoting cancer cell cycle arrest (Hayes et al., 2008) and selection for improved indolic profiles of rocket could lead to increased health benefits.

Temporal changes in 4MOB were most pronounced in RW2, with a large and significant increase in abundance at D7 ($15.6\,\mu mol\ g^{-1}$) compared to D0 ($2.5 \pm 0.7\,\mu mol\ g^{-1}$) and D4 ($3.3\,\mu mol\ g^{-1}$). Indolic GSLs are involved with abscisic acid (ABA) metabolism and synthesis (Malka and Cheng, 2017), and therefore such large increases over the course of shelf life may be indicative of increased senescence induced by plant hormone activity. This may be of significance when selecting rocket cultivars for improved shelf life traits; especially in second cut *D. tenuifolia*, which is the most common rocket product on global supermarket shelves.

Second cuts of each cultivar produced large average increases in GHP formation (Fig. 3). The patterns of change over shelf life did not match those of GSLs, suggesting that GSL content is not an accurate proxy for the abundance and ratios of GHPs that are formed. Importantly SF, which has been linked with anticarcinogenic effects *in vivo* (Liang et al., 2008) saw large increases in the *E. sativa* cultivars, but not in *D. tenuifolia*; concentrations were significantly higher in RS8 ($2.4 \pm 0.4\,\mu mol\ g^{-1}$; $P < 0.0001$). This was also repeated at D4 (RS8, $2.6 \pm 0.6\ \mu mol\ g^{-1}$; $P < 0.0001$, Table 3 & Supplementary Data File S1) and indicates that second cut *E. sativa* may be better suited for formation of health related SF at lower growth temperatures than *D. tenuifolia*.

3.2.3. First cut at 30 °C

In RS4 the decrease from D0 to D7 (Fig. 2) was predominantly due

Table 3
Results of Analysis of Variance type III sums of squares significance values for four rocket cultivars grown under three environmental temperature conditions, at three postharvest time points.

Factors & interactions		Glucosinolates							
	Time point / cultivar	Glucorucolamine	Glucoraphanin	Progoitrin	Diglucothiobeinin	Glucosativin	Glucoerucin	Dimeric 4-mercaptobutyl	Glucobrassicin
Cultivar	D0	***	*	ns	***	***	ns	***	ns
	D4	**	ns	ns	***	***	**	***	***
	D7	***	***	***	***	***	***	***	ns
Growth temperature	D0	ns	ns	ns	ns	ns	*	ns	ns
	D4	ns	ns	ns	ns	ns	ns	ns	***
	D7	ns	ns	***	ns	***	ns	ns	ns
Cut number	D0	**	ns	*	ns	ns	ns	ns	**
	D4	ns	ns	ns	ns	ns	ns	ns	***
	D7	ns	ns	**	ns	ns	ns	ns	ns
Cultivar x temperature	D0	**	ns	ns	ns	ns	ns	ns	*
	D4	ns	ns	**	ns	ns	ns	ns	***
	D7	*	ns	**	**	***	ns	ns	ns
Cultivar x cut number	D0	ns	ns	ns	ns	ns	ns	ns	ns
	D4	ns	ns	ns	ns	ns	ns	ns	***
	D7	ns	ns	***	ns	ns	ns	ns	ns
Cultivar x temperature x cut number	D0	*	ns	*	ns	ns	**	ns	*
	D4	ns	ns	***	ns	ns	ns	ns	***
	D7	ns	ns	***	ns	ns	ns	ns	ns
Temperature x cut number	RS4	*	ns	*	**	***	ns	ns	ns
	RS8	ns	*	ns	ns	ns	**	ns	**
	RW2	ns	ns	ns	ns	ns	ns	ns	ns
	RW3	ns	**	*	ns	ns	ns	ns	ns
Temperature x time point	RS4	ns	ns	ns	ns	***	ns	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns
	RW2	ns	ns	*	ns	ns	ns	ns	ns
	RW3	ns	ns	*	ns	ns	ns	ns	ns
Cut number x time point	RS4	ns	ns	ns	ns	**	*	ns	ns
	RS8	ns	ns	ns	ns	ns	ns	ns	ns
	RW2	ns	ns	ns	ns	ns	ns	ns	ns
	RW3	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x cut number x time point	RS4	ns	ns	ns	ns	**	ns	ns	ns
	RS8	ns	ns	ns	ns	*	ns	ns	ns
	RW2	**	ns	ns	**	*	ns	ns	ns
	RW3	ns	*	**	ns	ns	ns	ns	ns

Factors & interactions	Glucosinolates					Hydrolysis products			
	Gluconasturtiin	4-methoxy-glucobrassicin	Neoglucobrassicin	Total Glucosinolates	Sativin	Erucin	Sulforaphane	Bis(4-isothiocyanato-butyl)-disulfide	Total glucosinolate hydrolysis products
Cultivar	***	***	ns	***	***	ns	***	ns	***
	***	***	ns	***	***	***	***	ns	***
	**	***	ns	***	*	**	***	ns	**
Growth temperature	ns	**	ns	ns	ns	ns	ns	ns	*
	ns	**	ns	ns	ns	ns	ns	ns	ns
	ns	***	ns	ns	ns	ns	*	ns	ns
Cut number	ns	ns	*	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	***	ns	ns	ns	***
	ns	ns	ns	ns	***	ns	ns	***	***

(*continued on next page*)

Table 3 (*continued*)

Factors & interactions	Glucosinolates					Hydrolysis products			
	Gluconasturtiin	4-methoxy-glucobrassicin	Neoglucobrassicin	Total Glucosinolates	Sativin	Erucin	Sulforaphane	Bis(4-isothiocyanato-butyl)-disulfide	Total glucosinolate hydrolysis products
Cultivar x temperature	ns	**	ns	ns	ns	ns	**	ns	ns
	*	*	ns	ns	ns	ns	**	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cultivar x cut number	ns	ns	ns	ns	ns	ns	**	ns	*
	ns	ns	ns	ns	ns	ns	***	ns	*
	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cultivar x temperature x cut number	ns	ns	ns	ns	ns	ns	ns	ns	ns
	**	ns	ns	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x cut number	ns	ns	ns	**	ns	ns	ns	ns	ns
	ns	ns	ns	ns	**	ns	**	ns	**
	ns	***	**	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x time point	ns	ns	ns	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	*
	ns	***	**	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cut number x time point	ns	ns	ns	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns
	ns	***	**	ns	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns
Temperature x cut number x time point	ns	ns	ns	*	ns	ns	ns	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns
	ns	***	**	ns	ns	ns	**	ns	ns
	ns	ns	ns	ns	ns	ns	ns	ns	ns

Abbreviations: D0 = day 0 shelf life; D4 = day 4 shelf life; D7 = day 7 shelf life; RS = salad rocket, *Eruca sativa*; RW = wild rocket, *Diplotaxis tenuifolia*.
Significance values: *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$. See Supplementary Data File S1 for detailed P-values and Tukey's Honest Significant Difference pairwise comparisons.

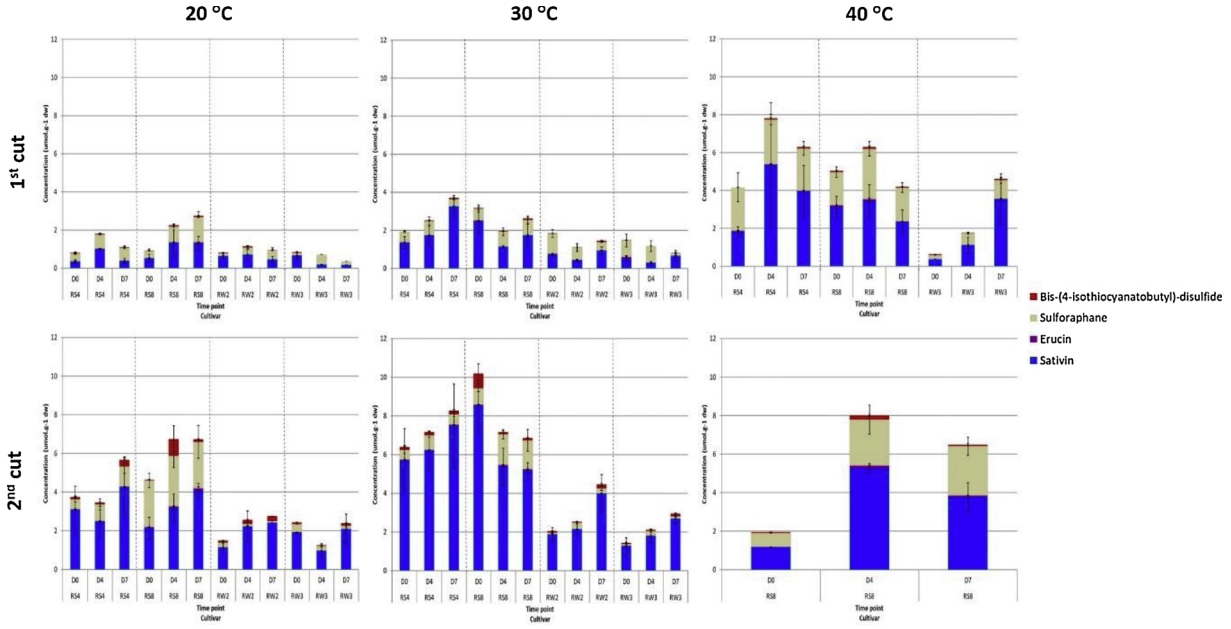

Fig. 3. Glucosinolate hydrolysis product concentrations produced by first and second cut *Diplotaxis tenuifolia* (RW) and *Eruca sativa* (RS) grown at four different temperatures. See inset for hydrolysis product colour coding. Abbreviations: D0, start of shelf life; D4, fourth day of shelf life; D7, seventh day and end of commercial shelf life. Error bars represent standard error of the mean for each respective compound. For detailed statistical and Tukey's HSD pairwise comparisons, see Supplementary Data File S1. See Table S1 for the numbers of replicates for each cultivar and treatment. Note that sample RW3 at 40 °C consists of < 3 biological replicates due to plant death.

to a significant reduction in GSV (from 20.4 ± 3.0 μmol g^{-1} to 0.9 ± 0.2 μmol g^{-1}; $P < 0.0001$, Table 3 & Supplementary Data File S1). This trend was not reflected in the GHP profile of RS4 however, where concentrations were highest at D7 (3.7 ± 0.6 μmol g^{-1}). Although no significant differences were found between cultivars or growth temperatures, these trends again suggest that GSL content is not an accurate predictor of GHPs.

3.2.4. Second cut at 30 °C

Second cuts showed no significant differences from the first at 30 °C for GSL content, indicating more consistent biosynthesis. This is a desirable characteristic for growers and processors as it in turn may contribute to improved consistency in taste and flavour between cuts.

In terms of individual GSL differences between cultivars, RS8 again contained significantly higher concentrations of 4MOB (8.9 ± 1.2 μmol g^{-1}) than RW3 at D0 (1.2 ± 0.2 μmol g^{-1}; $P < 0.0001$). On D4 of shelf life, RW3 by contrast contained significantly more PRO than the other cultivars (2.0 ± 0.5 μmol g^{-1}; $P < 0.0001$; Table 3 & Supplementary Data File S1). This compound is known to impart extreme bitterness and is a target for reduction through breeding (Ishida et al., 2014). While concentrations may not be high at the point of harvest, breeding selections should also take into account such possible increases in synthesis postharvest to reduce consumer rejection. PRO is also associated with anti-nutritional properties (Mithen et al., 2000) and efforts should be made to reduce concentrations in rocket cultivars through breeding.

In terms of temporal changes for each cultivar over the shelf life duration, one significant difference was of note: compared to the D0 sample point, D4 and D7 samples of RS4 contained significantly greater concentrations of GRA (5.7 ± 1.1 μmol g^{-1}, 10.6 ± 0.2 μmol g^{-1}, and 11.7 ± 1.3 μmol g^{-1}, respectively; $P < 0.0001$, Table 3 & Supplementary Data File S1). This matches observations made by Bell et al. (2017) where shelf life increases in this GSL were also observed for some cultivars.

Despite negligible changes in GSL content between cuts, GHPs saw large and significant increases compared to the first cut. The exact regulatory mechanisms for GHP formation in rocket are largely unknown, but evidence is mounting that it is not purely a spontaneous process of 1:1 conversion of GSLs. It may be that although GSL concentrations may be lower, myrosinase activity can remain high higher, and/or actively promote the formation of GHPs; such as through the action of ESM1 genes. It is important to understand how this is controlled under abiotic stress conditions, as it will likely influence the nutritional benefits obtained from leaves.

With the exception of RS8, all cultivar concentrations peaked on D7 further supporting previous reports of this (Bell et al., 2017c). At D0, RS8 produced significantly more SAT (8.5 ± 1.4 μmol g^{-1}; $P < 0.0001$) than the *D. tenuifolia* cultivars; and the highest concentrations overall of any tested sample (10.2 ± 2.0 μmol g^{-1}; $P < 0.0001$, Table 3 & Supplementary Data File S1). It is possible that such high concentrations of this compound would greatly increase the pungency of a cultivar, and support the anecdotal observations often made by growers.

3.2.5. First cut at 40 °C

RW3 contained significantly less GRM (3.5 μmol g^{-1}; $P < 0.0001$), GRA (5 μmol g^{-1}; $P = 0.000$), DGTB (not detected; $P < 0.0001$), and DMB (1.3 μmol g^{-1}; $P < 0.0001$) than the *E. sativa* cultivars at D0. In D4 samples, concentrations increased in RW3, however this was significantly lower than RS4 and RS8 for accumulation of GSV (1.3 ± 0.3 μmol g^{-1}; $P < 0.0001$). At D7, concentrations in RW3 declined, with RS4 and RS8 containing significantly more GRM, DGTB, and GSV (all $P < 0.0001$, Table 3 & Supplementary Data File S1).

Relative concentrations of GHPs were higher in RW3, particularly at D7, than the relative amounts of GSLs. The trend for the two *E. sativa* cultivars to contain higher abundances was however similar (Fig. 3). At D4 these contained significantly greater concentrations of SF than both RW2 RW3 ($P < 0.0001$, Table 3 & Supplementary Data File S1), but by D7 there were no significant differences. The retention of SF throughout the shelf life period is an important finding that suggests that potent health-related effects (Sivapalan et al., 2018) may be present in rocket leaves up to a week postharvest, even after the imposition of severe abiotic stress.

3.2.6. Second cut at 40 °C

RS8 was the only cultivar tested that survived and regrew under the

40 °C treatment, and it also contained the highest observed GSL concentrations of any condition or cut (Fig. 2). While no significant differences between each time point were observed, there is a clear trend for concentrations to increase at D7. This is one of the highest concentrations reported to-date for *E. sativa*, and it is clear that temperature response combined with cut in this cultivar results in extremely high GSL concentrations postharvest. GHPs however were relatively low (Fig. 3) with highest abundance at D4 (8.0 ± 1.1 μmol g^{-1}). This disparity between GSL and GHP abundances may be suggestive of myrosinase impairment or reduced activity.

3.2.7. Cultivar differences between growth temperature treatments

The disparity between rocket species GSL accumulations and GHPs is also evidenced when comparing broadly between growth temperature conditions. Table 3 contains the Type III Sum of Squares analysis results and reveals there are fewer significances between growth temperature and cut treatments for *D. tenuifolia* than *E. sativa* cultivars. This suggests that the former species is much less variable in terms of GSLs and GHPs, however (based on the two cultivars tested) is unable to achieve significant changes in health and flavour-related compounds between growth temperatures. This attribute is important however, and better for (potentially) maintaining uniformity of taste and flavour traits between temperature extremes. If the goal is to make rocket species more nutritionally dense, *E. sativa* possesses a degree of environmental plasticity in response to different growth temperatures that lends itself well to synthesis of GSLs such as GRA and GER. RS4 and RS8 also produced greater concentrations of SF under each condition and cut, therefore making cultivars more efficacious against chronic diseases than the more commonly consumed *D. tenuifolia*.

Considering the factors contributing to differences in concentrations, *E. sativa* is significantly influenced by temperature, with relatively few compounds affected by the respective interactions between temperature, harvest (cut) and sample point. For RW2 indolic GSL concentrations (4MOB and NGB) were most significantly affected by each factor and their interactions. RW2 by contrast had the most variability for GRA and PRO concentrations. GHPs by contrast (regardless of species) were most significantly influenced by the cut number. This indicates that while total GSLs may not be significantly changed after second cut, GHPs are. This may reflect a change in the expression of respective genes and enzymes regulating hydrolysis rather than those involved in GSL biosynthesis *per se*, and may give rise to improvements in nutritional quality.

3.2.8. Effects of growth temperature on postharvest concentrations

Irrespective of cultivar or species, several significant associations between growth temperature and shelf life concentrations of GSL compounds. At D0 GER concentrations were significantly affected by growth temperature ($P = 0.012$), as well as total GHPs ($P = 0.027$). At D4, two significances were observed for the indolic GSLs GBR ($P = 0.001$) and 4MOB ($P = 0.002$). By D7 there were several GSLs and one ITC significantly associated with growth temperature; these were PRO ($P < 0.0001$), GSV ($P = 0.000$), 4MOB ($P = 0.000$), and SF ($P = 0.034$, Table 3 & Supplementary Data File S1). These data are of particular interest for two reasons: the first is that PRO and GSV are thought to contribute significantly to the taste and flavour profile of rocket leaves (Pasini et al., 2011; Raffo et al., 2018). Their relative increases/decreases over the course of shelf life may therefore alter sensory properties, and conceivably consumer preference (Bell et al., 2017b). The second is that SF is associated with health-related benefits, and therefore cultivars could be improved by selecting for plants able to form greater concentrations later into shelf life (*e.g.* RS8; Bell, Yahya, et al., 2017).

As presented in Table 3, there were numerous significant interactions between cultivar, cut, and growth temperature, making exact predictions of postharvest concentrations and profiles difficult. It is clear however that growth temperature influences the potential nutritional and sensory status of cultivars, and goes some way to explain the inconsistencies observed by growers and processors between growing regions and cuts of the same cultivar.

3.3. Principal component analysis

Fig. 4 shows PCs 1 and 2 of the PCA analysis and explain 40.87 % of the observed variation within the data. PC1 separates predominantly

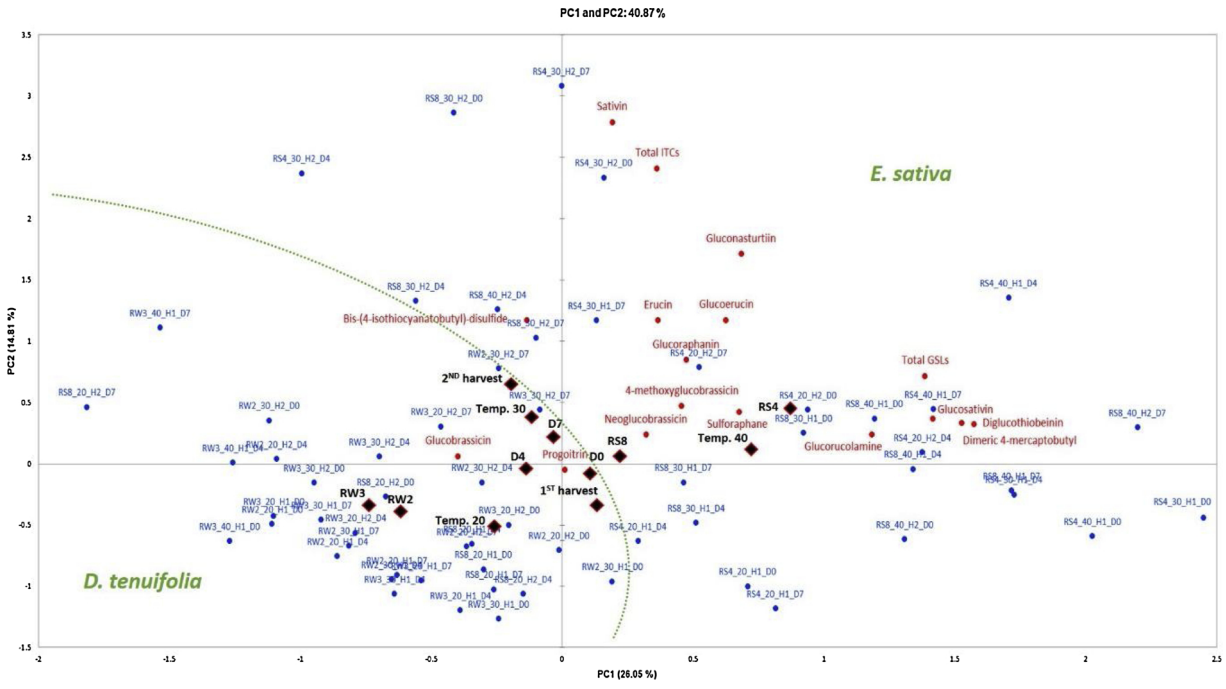

Fig. 4. Principal Component Analysis biplot of glucosinolate and hydrolysis product concentrations or rocket cultivars grown at four different growth temperatures. Components PC1 and PC2 are presented and represent 40.87 % of total variation. Blue data points = sample loadings; red data points = glucosinolate and hydrolysis product scores; black diamonds = cultivar, cut, shelf life time point, and temperature centroids (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

for total GSL content, as well as DGBT, GSV and DMB. PC2 by comparison separates strongly for SAT and total GHP formation. This is of note because it indicates that a high GSL concentration does not necessarily correlate with high GHP formation. To give two examples; first cut D0 RS4 plants grown at 30 °C contained relatively reduced concentrations of GHPs compared to the observed GSLs. Conversely, second cut plants of the same cultivar and temperature saw marked increases in GHP formation relative to GSL concentration, which was largely unchanged between cuts at this temperature.

PC1 also separates for species (Fig. 4), as it is clear that the *D. tenuifolia* cultivars tested are generally low accumulators of GSLs, and form relatively few GHPs compared to RS4 and RS8. As a proportion of the overall GSL profile, RW2 and RW3 contained greater concentrations of indolic GSLs (such as GBR) as well as PRO.

As highlighted in previous studies however, concentrations of GSLs/GHPs are not in and of themselves indicators of perceived sensory traits such as pungency; so it may be that the stark differences in the species' profiles may not be reflected in their taste and flavour attributes (Bell et al., 2017a). Other modulating influences such as sugar concentrations may also affect this, so it is therefore important to note that pungency is not indicative of health-related benefits and *vice versa*. A salad rocket may, for example, be very mild tasting but still potentially contain many fold-higher amounts of GHPs which are masked by other compounds.

4. Conclusions

This paper has demonstrated the effects of cultivation temperature and multiple harvests on postharvest GSL and GHP concentrations in rocket species. While it has been anecdotally accepted by growers that pungency increases according to the number of cuts a crop receives, very few previous studies have accounted for this common horticultural practice.

Temperate grown crops (~20 °C average outdoor summer temperatures) are often noted for their less pungent aroma and flavour than those from hotter countries (such as Italy, Portugal, and Morocco). Our data show that total GSL concentrations between growth temperatures are not significantly affected, but that it is the abundance of GHPs produced which differs. It is clear and unsurprising that growth at 40 °C is detrimental to plant development and regrowth, however it is also apparent that there is a significant increase in GSL biosynthesis, and also SF formation postharvest. Our data also highlight that some *E. sativa* cultivars may be better adapted to growth under extreme temperatures, as RS8 showed remarkable tolerance to the 40 °C treatment, and a propensity for increased SF productions under these conditions. More research will be required to determine if this tolerance is indicative of the species more widely when compared with *D. tenuifolia*.

Rocket crop growth under protected conditions can routinely reach or even exceed 40 °C, especially in summer months in countries such as Italy. With such extremes in temperature likely to increase in future due to climate change, it is important to determine the effects on nutritionally dense crops such as rocket. It is likely under such conditions that yields and production will be reduced, but that the nutritional density of crops may actually increase.

The GSL data presented are in agreement with previous studies of other Brassicaceae species (see Bell and Wagstaff, 2017 for a summary) however few other studies have also analysed GHPs in tandem. Our data suggest that it is incorrect to assume that GHP profiles and abundances are affected in a similar fashion to GSLs under different growth temperatures. Fluctuations in GHP abundance and conversion from GSLs is related to both environment and genotype. This is consistent with observations found in *Brassica* vegetables, where the concentrations of hydrolysis products is typically much less than the total concentration of the GSL precursors (Hanschen and Schreiner, 2017). GRA conversion to SF varied from 0.9 % (RW2, second cut, D7, 20 °C) to 25.1 % (RS8, second cut, D4, 40 °C); and GSV/DMB conversion to SAT (Fechner et al., 2018) from 1.7 % (RS4, first cut, D7, 20 °C) to 100 % (RW3, first cut, D7, 40 °C).

The relative changes in the formation of these compounds between growth temperatures indicates that there is an environmental effect upon myrosinase. This may be in terms of total plant content and/or activity, but the differences observed here between genotypes suggests that this is also as a result of genetic variation. The focus of breeding should therefore shift from selecting cultivars with high GSL concentrations, and more towards those that convert them to GHPs most efficiently. This may involve selection for different myrosinase genotypes, but could also feasibly extend to epithiospecifier modifier proteins, such as ESM1, which promote ITC formation (Angelino and Jeffery, 2014).

There are many factors regulating and inhibiting hydrolysis of GSLs other than nascent myrosinase abundance and activity; such as pH, temperature, ascorbic acid concentration, and enzyme co-factor presence/absence. While the kinetics of isolated compounds and myrosinase are well understood, it is still unclear how regulatory mechanisms within plant matrices control GHP formation and abundance. It is important to better understand the postharvest hydrolysis of GSLs since any health-related impacts of consuming Brassicaceae foods (such as rocket) will depend greatly upon pre-harvest environment, rather than postharvest storage conditions alone.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jake Jasper: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Carol Wagstaff:** Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Luke Bell:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2020.111157.

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